FORM PTO-1390 (REV 10-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 211-213

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 10/019388 To Be Assigned

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB00/02473	28 June 2000 (28.06.2000)	28 June 1999 (28.06.1999)

TITLE OF INVENTION: NEW INDOLOCARBAZOLE ALKALOIDS FROM A MARINE ACTINOMYCETE

APPLICANT(S) FOR DO/EO/US: Garcia Gravalos, Dolores et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☐ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Copy of the International Preliminary Examination Report; Copy of the Written Opinion; and Return Receipt Postcard

531 Rec'd PCT/PTO 28 DEC 2001

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/019388 To Be Assigned	PCT/GB00/02473	211-213

17. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	18 - 20 =	0	X **$18.00**	$ 0.00	
Independent claims	1 - 3 =	0	X **$80.00**	$ 0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$	

TOTAL OF ABOVE CALCULATIONS =	$ 890.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.	$	
SUBTOTAL =	$	
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$	
TOTAL NATIONAL FEE =	$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$	
TOTAL FEES ENCLOSED =	$ 890.00	
	Amount to be refunded:	$
	charged:	$

a. ☒ A check in the amount of $ 890..00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 07-1730 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 07-1730. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

RUBENSTEIN, Allen I.
GOTTLIEB RACKMAN & REISMAN PC
270 Madison Avenue
New York, NY 10016-0601
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SIGNATURE: Allen Rubenstein

Allen I. RUBENSTEIN
NAME

27,673
REGISTRATION NUMBER

Docket No. 211-213

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Dolores Garcia Gravalos et al. **Group Art Unit**: To Be Assigned

Serial No. : To Be Assigned **Examiner** : To Be Assigned

Filing Date : Enclosed herewith

For : NEW INDOLOCARBAZOLE ALKALOIDS FROM A MARINE ACTINOMYCETE

Commissioner for Patents
Box PCT
Washington, D.C. 20231
Attention: DO/EO/US

PRELIMINARY AMENDMENT

Sir:

Prior to the substantive examination of the subject application please amend thereof as follows:

In the claims:

Please amend claims 12 and 15-18 as follows. (A marked-up version of the claims is presented on pages 1-2 of the Appendix 1attached herewith).

12 (Amended). A process for the production of a compound of formula (1) as define in claim 1, or a pharmaceutically acceptable salt thereof, comprising cultivating a strain of a microorganism capable of producing a compound of formula (1), recovering the compound of formula (1) from

the cultured broth , and, optionally, salifying the recovered compound.

15 (Amended). A pharmaceutical composition containing as an active ingredient a compound of formula (1) as define in claim 1, or a pharmaceutically acceptable salt thereof, in conjunction with a pharmaceutically acceptable carrier or diluent.

16 (Amended). A compound of formula (1) as defined in claim 1, or a pharmaceutically acceptable salt thereof for use as a medicament.

17 (Amended). The use of a compound of formula (1) as defined in claim 1, or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment or prophylaxis of malignant tumours in a mammal.

18 (Amended). A method for the treatment or prophylaxis of malignant tumours in a mammal, comprising administering to a mammal in need of such treatment an effective amount of a compound of formula (1) a s defined in claim 1, or a pharmaceutically acceptable salt thereof.

REMARKS

Claims 12 an 15-18 have been amended to eliminate their multiple dependency.

It is respectfully submitted that no new matter has been added by aforementioned

amendment and entry thereof is earnestly solicited.

No fee is believed necessary in connection with the filing of this Amendment. However, if any fee is deemed necessary, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 07-1730, under Docket No. 211-213. A duplicate copy of this communication is attached for that purpose.

Respectfully submitted
GOTTLIEB, RACKMAN & REISMAN, P.C.

Dated: 12/28/01

By: _____
Allen I. Rubenstein
Attorney for applicants
Reg. No. 27,673

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APPENDIX 1

MARKED-UP VERSION OF THE CLAIMS

12 (Amended). A process for the production of a compound of formula (1) as define in [any one of claims] claim 1 [to 11], or a pharmaceutically acceptable salt thereof, comprising cultivating a strain of a microorganism capable of producing a compound of formula (1), recovering the compound of formula (1) from the cultured broth , and, optionally, salifying the recovered compound.

15 (Amended). A pharmaceutical composition containing as an active ingredient a compound of formula (1) as define in [any one of claims] claim 1 [to 11], or a pharmaceutically acceptable salt thereof, in conjunction with a pharmaceutically acceptable carrier or diluent.

16 (Amended). A compound of formula (1) as defined in [any one of claims] claim 1 [to 11], or a pharmaceutically acceptable salt thereof for use as a medicament.

17 (Amended). The use of a compound of formula (1) as defined in [any one of claims] claim 1 [to 11], or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment or prophylaxis of malignant tumours in a mammal.

18 (Amended). A method for the treatment or prophylaxis of malignant tumours in a mammal, comprising administering to a mammal in need of such treatment an effective amount of a

compound of formula (1) a s defined in [any one of claims] claim 1 [to 11], or a pharmaceutically acceptable salt thereof.

New Indolocarbazole Alkaloids from a Marine Actinomycete

FIELD OF THE INVENTION

New indolocarbazole alkaloids have been isolated from the culture broth of a staurosporine-producing actinomycete (CLCO-002). Their production by aerobic fermentation under controlled conditions of the strain, and the isolation and purification of compounds are described herein. The compounds and the fermentation broth demonstrate significant activity against several cancer cell lines.

BACKGROUND OF THE INVENTION

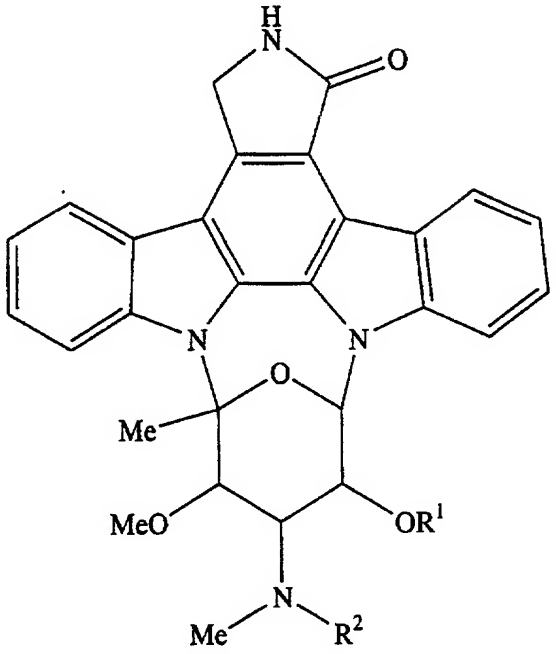
The isoenzyme family of protein kinase C (PKC) plays a key role in signal transduction and cellular regulation (Y. Nishizuka, 1988). From the observation that the tumor promoting phorbol esters are able to stimulate PKC activity (Y. Nishizuka, 1984), it was concluded that inhibitors of this enzyme could be useful for cancer chemotherapy. PKC inhibitors have been extensively investigated as potential drugs for the treatment of cancer. Accordingly, a goal of the present invention is to provide new antitumor agents; these compounds are alkaloids with significant activity against several cancer cell lines.

Yet another objective of this invention is to provide pharmaceutical compositions for administering to a patient in need of treatment using the active compounds described herein.

Microbial products are interesting because their industrial production is well established at present times. Therefore, another objective of this invention is directed to the production of the active compounds and to their isolation and purification from the resulting fermentation broth.

SUMMARY OF THE INVENTION

This invention provides compounds of formula (1).



(1)

wherein:

R^1 is a hydrogen atom, an alkyl group having 1 to 6 carbon atoms or an alkoxy group having 1 to 6 carbon atoms; and

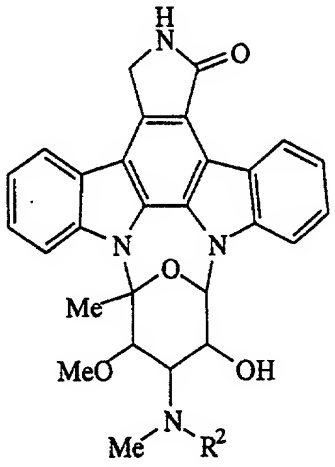
R^2 is a hydrogen atom, an alkyl group having 1 to 6 carbon atoms or an alkoxy group having 1 to 6 carbon atoms;

and pharmaceutically acceptable salts thereof.

In the definitions of the groups R^1 and R^2 in formula (1), the alkyl groups and the alkyl moiety of the alkoxy groups are a straight or branched chain alkyl group having 1 to 6 carbon atoms such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, neopentyl and hexyl.

It is preferred that R^1 and R^2 independently represent a hydrogen atom or an alkyl group having from 1 to 4 carbon atoms, particularly a hydrogen atom, a methyl group or an ethyl group.

In a particularly preferred embodiment. the present invention relates to 4'-N-methyl-5'-hydroxystaurosporine (IB-97224) and 5'-hydroxystaurosporine (IB-97225), with structural formulae:



IB-97224 (R^2=Me)

IB-97225 (R^2=H)

In this invention the process of obtaining compounds of formula (1) or a pharmaceutically acceptable salt thereof is also described. The process comprises cultivating a strain of a microorganism capable of producing a compound of formula (1), recovering the compound of formula (1) from the cultured broth, and, optionally, salifying the recovered compound.

An especially preferred process for producing compounds IB-97224 and IB-97225 comprises cultivating a strain of a microorganism capable of producing IB-97224 and IB-97225 in an aqueous nutrient medium with assimilable carbon and nitrogen sources and salts, under controlled submerged aerobic conditions. The compounds IB-97224 and IB-97225 are recovered and purified from the cultured broth.

The preferred culture is strain CLCO-002, and its chemical, biochemical and morphological characters show that it belongs to the *Actimomicetales* group. Other actinomycete strains may also be used in the process according to the invention.

As described above, the compounds of formula (1), especially IB-97224 and IB-97225, have been found to have good activity against murine and human tumor cell lines, including P-388D_1, HT-29, A-549 and SK-MEL-28.

Therefore, the invention also provides a method for the treatment or prophylaxis of malignant tumours in a mammal, comprising administering to a mammal in need of such treatment an effective amount of a compound of formula (1) as defined above or a pharmaceutically acceptable salt thereof.

The invention further relates to the use of a compound of formula (1), as defined above, or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment or prophylaxis of malignant tumours in a mammal.

The present invention also relates to pharmaceutical preparations which contain as an active ingredient compounds of formula (1), or a pharmaceutically acceptable salt thereof, together with a pharmaceutically acceptable carrier or diluent as well as the processes for its preparation.

Examples of pharmaceutical compositions include any solid (tablets, pills, capsules, granules, etc.) or liquid (solutions, suspensions or emulsions) with suitable composition for oral. topical or parenteral administration, and they may contain the pure compounds or in combination with any carrier or other pharmacologically active compounds. These compositions may need to be sterile when administered parenterally.

The correct dosage of a pharmaceutical composition of will vary according to the particular formulation, the mode of application, and the particular *situs*, host and bacteria or tumor being treated. Others factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of the disease shall be taken in account. Administration can be carried out continuously or periodically within the maximum tolerated dose.

DETAILED DESCRIPTION OF THE INVENTION

The Producing Organism

The microorganism utilised for the production of these new compounds is preferably an actinomycete strain, particularly actinomycete strain CLCO-002, a culture of which has been deposited in the Colección Española de Cultivos Tipo at the University of Valencia, Spain under the accession number CECT-3347. This deposit has been made under the provisions of the Budapest Treaty and all restrictions on the availability thereof to the public will be irrevocably maintained upon the granting of a patent on this application.

The organism was isolated from an unidentified marine sponge collected in Canary Islands waters.

All cultures were incubated at 27°C and records of results were made weekly up to 21 days.

A description of the organism is as follows:

Morphology

The culture media utilised for this study were, ISP media No 2, 4, 5 and 6 (Shirling and Gotlieb, 1966), ATCC medium No 172 (American Type Culture Collection Catalog, 1989), Czapek Agar (Atlas, 1993), Bennet Agar (Atlas, 1993), 1.5% Water Agar (Luedemann). All media were supplemented with 50% artificial seawater. After 21 days at 28°C growth was studied. Several shades of orange were observed. No aerial mycelium was formed. Substrate mycelium was branched. No soluble pigment was observed.

Physiological characteristics

For carbon and nitrogen utilization studies ISP-9 was used (Shirling & Gotlieb, 1966). Due to low growth rate of CLCO-002 under defined media, the carbon and nitrogen utilisation tests showed residual growth so no clear results could be obtained. NaCl resistance was determined by using ATTC's 172 medium containing increasing concentrations of NaCl. The optimal concentration of salt was 1%. No growth was observed with 7% salt.

Cell chemical composition

Aminoacids:

Diaminopimelic acid was determined by the method of Hasegawa et al. (1983). The *meso*-2,6-Diaminopimelic acid isomer was present in the whole cell hydrolysate of strain CLCO-002.

Fatty acids:

FAMEs were determined by the method of Van der Auwera et al. (1986). The FAME composition as well as comparison with other similar strains is described in Table 1.

While the deposited organism is clearly preferred, the present invention is not restricted or limited to this particular strain or organisms. It is the intention of the present inventors to include any other producing organisms, strains or mutants within the scope of this invention.

TABLE 1

FAME composition of strain CLCO-002 and other actinomycete strains. Composition is given as percentage of total fatty acids content.

	13:0	i-14:0	14.0	i-15:0	a-15:0	15:0	i-16 1	i-16:0	16:1	16:0	i-17:1	i-17.0	a-17.0	17 1	17:0	i-18:1	i-18:0	cis-18:1	18:0
CLCO-002	< 1	< 1	< 1	16.91	3 94	6 71	< 1	31.83	< 1	< 1	3.73	< 1	< 1	24.33	3.31	< 1	< 1	4.13	< 1
STALBUS	< 1	6.52	< 1	9.88	22.92	< 1	5 50	25.29	< 1	3.75	1.28	3 38	8 60	< 1	< 1	< 1	1.09	< 1	< 1
SPAMETH	1.21	10.34	< 1	1.86	< 1	4.30	< 1	15.51	5.63	8.62	1.08	< 1	< 1	24.02	9.43	7.11	< 1	4.60	1.04
SPVIRIDO	< 1	4 04	1.10	18.94	2.71	4.89	< 1	26.44	< 1	4.43	< 1	2.60	1.58	11 36	8.58	7.48	< 1	< 1	1.16
AMCITRE	< 1	< 1	3.18	< 1	< 1	1.03	< 1	6.37	12.62	40	< 1	< 1	< 1	< 1	1.16	< 1	< 1	14.25	2.82
APBRAZIL	< 1	3.15	< 1	15.46	18.91	2.76	< 1	19.07	2.15	1.79	< 1	2.39	9.64	11.18	2.82	< 1	< 1	3.38	1.06
AMPDIGIT	< 1	11.57	< 1	11.21	9.96	< 1	2.87	34.23	< 1	1 08	< 1	1.28	5.08	4 39	1.64	< 1	1.76	7.60	1 54
AMYORIE	< 1	3.40	2.37	19.94	4.66	1.17	< 1	11.85	5.59	18.41	< 1	2.99	4.44	3.09	2.73	< 1	< 1	6.21	3.04
MNCHALC	< 1	1 68	< 1	8 91	2.29	1.53	1.15	38.23	< 1	1 88	1 49	2.32	2.25	5.43	6.95	14 58	1.31	1.28	2.68
MNECHCA	< 1	1 17	< 1	6.97	1.24	2 81	< 1	30.88	< 1	2.29	1.63	4.11	1.68	12 15	4.90	7.23	< 1	10.05	1.69
MNFUSCA	< 1	< 1	< 1	26.56	6.53	< 1	< 1	8.58	< 1	< 1	7.30	11.89	13.25	2.90	3.37	3.59	< 1	2.33	1.94
SACCAER	< 1	3.06	1.35	14.41	8.62	1.04	5.68	20.07	13.84	6.16	4.55	2.20	5.31	2.02	< 1	< 1	< 1	< 1	1.43
NOAFRI	1.51	5.43	3.35	4.62	< 1	7.46	3.09	22.18	2.69	5.15	2.35	< 1	< 1	8.15	4.75	17.03	< 1	< 1	1.23
MTSALMO	< 1	1.12	1.28	6.75	< 1	7.83	7 53	21.58	1.21	1.97	1.01	< 1	1.07	11 58	5.53	17.34	< 1	< 1	< 1
MTRUBRA	< 1	1.40	1.38	4 12	< 1	3.41	7.27	25.00	2.63	3.89	2.17	1.08	< 1	6.84	4.97	15.44	1.25	< 1	1.61
MTROSEO	2.03	3.65	5.14	3.86	< 1	9.03	3.02	12.31	3.46	6.95	1.17	< 1	< 1	13.51	4.46	18.67	< 1	1.77	< 1
AMROSEO	< 1	2.19	1.24	6.73	1.09	6.94	1.43	22.21	2.21	3.61	2.74	1.03	< 1	10.97	4.33	17.84	< 1	< 1	< 1
MTFERRU	1.03	1.91	1.19	1 94	< 1	6.43	4.12	21.50	2.32	2.34	< 1	< 1	< 1	23.51	5.71	12.15	1.27	1.43	< 1

CLCO-002 = strain CLCO-002; AMCITRE = *Actinomadura citrea* DSM 43461; AMPDIGIT = *Ampullariella digitata* ATCC 15349; AMROSEO = *Actinomadura roseoviolacea* DSM 43144; AMYORIE = *Amycolatopsis orientalis* DSM 40040; APBRAZIL = *Actinoplanes braziliensis* ATCC 25844; MNCHALC = *Micromonospora chalcea* ATCC 31395; MNECHCA = *Micromonospora echinospora calichinensis* NRRL 15839; MNFUSCA = *Micromonospora fusca* NRRL B-3298; MTFERRU = *Microtetraspora ferruginea* DSM 43553; MTROSEO = *Microtetraspora roseola* ATCC 33579; MTRUBRA = *Microtetraspora rubra* ATCC 27031; MTSALMO = *Microtetraspora salmonea* ATCC 33580; NOAFRI = *Nocardiopsis africana* DSM 43748; SACCAER = *Saccharothrix aerocolonigenes* NRRL B-3298; SPAMETH = *Streptosporangium amethystogenes* DSM 43179; SPVIRIDO = *Streptosporangium viridogriseum* ATCC 25242; STALBUS = *Streptomyces albus* DSM 40313

Fermentation

Strain CLCO-002, when cultured under controlled conditions in a suitable medium produces the compounds IB-97224 and IB-97225. This strain is grown in an aqueous nutrient medium. under aerobic and mesophilic conditions, preferably between 22°C and 35°C at a pH ranging between 6.0 and 8.0. A wide variety of liquid culture media can be utilised for the cultivation of the organism. useful media are those that include an assimilable carbon source, such as starch, dextrin, sugar molasses, glycerol, glucose and the like, an assimilable nitrogen source such as proteins, protein hydrolysates, defatted meals, corn steep, and the like, and useful inorganic anions and cations such as sodium, magnesium, potassium, ammonium, sulphate, chloride, phosphate. carbonate, and the like. Trace elements may be added also. Aeration is preferably achieved by supplying air to the fermentation medium. Agitation is provided by a mechanical impeller. Conventional fermentation tanks have been found to be well suited for carrying out the cultivation of this organism. The addition of nutrients and pH control as well as antifoaming agents during the various stages of fermentation may be needed for increasing production and avoid foaming.

The required steps needed for production of these compounds by the preferred organism are:

Start with frozen or lyophilised mycelium. Obtain mycelial mass culturing the initial cells in shake flasks with a culture medium containing some of the ingredients described above at mesophilic temperatures and in aerobic conditions, this step may be repeated several times, as needed, and the material collected will be used as an inoculum to seed one or several fermentation tanks with any appropriate culture medium, if desired these tanks can be utilised also as inoculum, and this step can be repeated several times when needed, or they can serve as the production stage, depending on the broth volume needed. The production stage can last from very few days to more than one week, depending on strain, inoculum stages, temperature and other conditions. Once the fermentation has reached its maximum yield can be harvested for the isolation of the new compounds.

Production medium may be different than that used as inoculum. In Table 2 typical media are described that can be used for inoculum and production of these new compounds:

TABLE 2

Inoculum medium (g/litre)		Production medium (g/litre)	
Dextrose	5	Dextrose	5
Starch	20	Dextrin	20
Beef extract	3	Soybean meal	3
Yeast extract	5	Yeast extract	5
Peptone	5	Peptone	1
$CaCO_3$	4	$CaCO_3$	4
NaCl	4	NaCl	5
Na_2SO_4	1	Na_2SO_4	2.5
KCl	0.5	KCl	0.5
$MgCl_2$	2	$MgCl_2$	0.5
K_2HPO_4	0.5	K_2HPO_4	0.5
		$(NH_4)_2SO_4$	0.5
Tap water to 1 000 ml			

Production of these compounds can be monitored by whole broth assay against A-549 or any other sensitive cell or by HPLC or any other method with enough sensitivity.

Isolation of IB-97224 and IB-97225

Alkaloids IB-97224 and IB-97225 can be isolated from the mycelia cake by extraction with a suitable mixture of solvent such as $CHCl_3$:CH_3OH:H_2O. The activity is concentrated in the lower layer. The extracts from two repeated extractions can be combined and evaporated to dryness *in vacuo*.

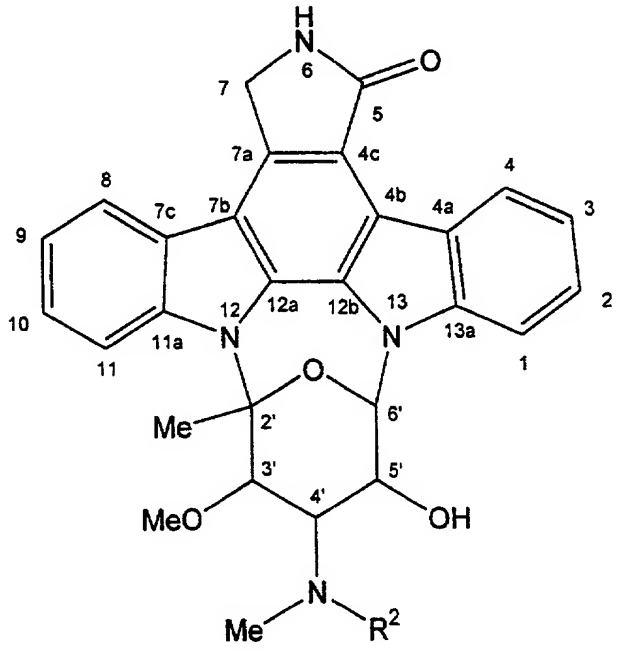
Separation and purification of IB-97224 and IB-97225 from the crude active extract can be performed by the use of the proper combination of conventional chromatographic techniques.

Fractionation can be guided by the antitumor activity of fractions, or by TLC visualized with vanillin in conc. H_2SO_4, or analytical HPLC with photodiode-array detector. HPLC analysis are performed at room temperature (Waters RCM 8x10, 8C18 10μm cartridge) using as mobile phase acetonitrile-sodium hydrogenphosphate 0.025M pH=3 (75:25) and a flow rate of 2 ml/min. and plotted at 290 nm. Compounds of interest showed retention times of 3.92 and 3.29 minutes to IB-97224 and IB-97225 respectively.

The spectral data given below enables the compounds to be identified as IB-97224 and IB-97225. The various atoms are numbered using the numbering system indicated below. The following abbreviations are used:

IR spectra: w: weak; m: medium; s: strong; br: broad.

NMR spectra: s: singlet; d: doublet; t: triplet; dd: doublet of doublets.



4'-N-methyl-5'-hydroxystaurosporine (IB-97224) (R^2=Me)

IR (KBr) ν_{max}/cm^{-1}: 3406 (s, br), 3070 (m), 2925 (s), 2852 (m), 1915 (w, br), 1664 (s), 1583 (s), 1450 (m), 1415 (m), 1391 (s), 1351 (s), 1319 (s), 1281 (s), 1249 (s), 1236 (m), 1223 (m), 1181 (m), 1150 (m), 1117 (s), 1103 (s), 1066 (s), 1018 (m), 988 (m), 887 (w), 835 (w), 816 (w), 742 (s), 698 (w), 664 (w), 636 (w), 609 (w).

^{1}H NMR (300 MHz, $CDCl_3$), δ/ppm: 9.43 (1H, d, J 7.7 Hz, C4H), 7.90 (1H, d, J 7.7 Hz, C8H), 7.76 (1H, d, J 7.7 Hz, C11H), 7.64 (1H, d, J 7.7 Hz, C1H), 7.53 (1H, t, J 7.7 Hz, C2H), 7.45 (1H, t, J 7.7 Hz, C10H), 7.38 (1H, t, J 7.7 Hz, C3H), 7.34 (1H, t, J 7.7 Hz, C9H), 6.52 (1H, s, C6'H), 6.50 (1H, s, N6H), 4.99 (1H, s, C7H), 4.43 (1H, d, J 9.9 Hz, C5'H), 3.95 (1H, s, C3H), 3.02 (1H, d, J 9.9 Hz. C4'H), 2.48 (3H, s, CH_3), 2.37 (6H, s, N4'(CH_3)$_2$), 2.03 (3H, s, CH_3O).

^{13}C NMR (75 MHz, $CDCl_3$), 173.65 (C5), 137.86 (C11a), 137.12 (C13a), 131.94 (C7a), 130.64 (C12a), 126.79 (C12b), 126.13 (C4), 125.46 (C2), 124.94 (C10), 124.54 (C7c), 123.22 (C4a), 121.49 (C8), 120.43 (C9), 119.98 (C3), 118.89 (C4c), 115.86 (C4b), 114.14 (C7b), 111.46 (C11), 108.97 (C1), 94.92 (C2'), 91.54 (C6'), 79.30 (C3'), 69.50 (C5'), 66.75 (C4'), 58.36 (CH_3O), 45.79 (C7), 41.67 (N4'(CH_3)$_2$), 28.00 (CH_3).

UV (75:25 CH_3CN / 0.025 M Na_2HPO_4 pH 3), λ_{max}/nm: 370, 354, 334, 320, 291, 242, 206.

m/z (Fast Atom Bombrdment) 497.2 (MH^+).

5'-Hydroxystaurosporine (IB-97225) (R^2=H)

IR (KBr) ν_{max}/cm^{-1}: 3415 (s, br), 3070 (m), 2931 (m), 2851 (m), 1991 (w, br), 1664 (s), 1583 (m), 1453 (s), 1416 (m), 1392 (m), 1352 (s), 1317 (s), 1280 (m), 1248 (m), 1236 (m), 1225 (m), 1151 (m), 1130 (m), 1118 (m), 1064 (m), 1036 (m), 1017 (m), 973 (w), 927 (w), 896 (w), 860 (w), 836 (w), 814 (w), 772 (m), 746 (s), 651 (w), 638 (w).

^{1}H NMR (300 MHz, $CDCl_3$), δ/ppm: 9.40 (1H, d, J 7.4 Hz, C4H), 7.89 (1H, d, J 7.4 Hz, C8H), 7.85 (1H, d, 7.4, C11H), 7.53 (1H, d, J 8.1 Hz, C1H), 7.44 (2H, t, J 7.4 Hz, C2H & C10H), 7.31 (2H, t, J 7.4 Hz, C3H & C9H), 6.49 (1H, d, J 1.2 Hz, C6'H), 6.43 (1H, s, N6H), 4.98 (1H, s, C7H), 4.26 (1H, dd, J 6.8 Hz, 1.2 Hz, C5'H), 4.14 (1H, d, J 2.8 Hz, C3'H), 3.09 (1H, dd, J 6.8 Hz, 2.8 Hz, C4'H), 2.71 (3H, s, CH_3O), 2.45 (3H, s, CH_3), 2.17 (3H, s, CH_3N4').

^{13}C NMR (75 MHz, $CDCl_3$), δ/ppm: 173.81 (C5), 138.86 (C11a), 137.05 (C13a), 132.17 (C7a), 130.50 (C12a), 126.89 (C12b), 126.13 (C4), 125.33 (C2), 124.67 (C10), 124.52 (C7c), 123.24 (C4a), 121.01 (C8), 120.32 (C9), 119.92 (C3), 118.56 (C4c), 115.64 (C4b), 114.19 (C7b), 113.50 (C11), 108.10 (C1), 92.37 (C2'), 88.38 (C6'), 80.14 (C3'), 70.03 (C5'), 60.11 (C4'), 59.02 (CH_3O), 45.88 (C7), 33.68 (CH_3N4'), 28.96 (CH_3).

UV (75:25 CH_3CN / 0.025 M Na_2HPO_4 pH 3), λ_{max}/nm: 370, 354, 334, 320, 291, 242, 206.

m/z (Fast Atom Bombardment) 483.2 (MH^+).

Biological activity

The antitumor activities of IB-97224 and IB-97225 have been determined *in vitro* in cell cultures of mouse leukemia P-388D_1, human lung carcinoma A-549, human colon carcinoma HT-29 and human melanoma SK-MEL-28. The procedure was carried out using the methodology described by Bergeron, et al. (1984), and by Schroeder, et al. (1981).

The present invention will be further illustrated with reference to the following examples which aid in the understanding of the present invention, but which are not to be construed as limitations thereof. All percentages reported herein, unless otherwise specified, are presented by weight. All temperatures are expressed in degrees Celsius. All incubations are carried out at 28 °C and flasks are shaken in an orbital shaker. All media and recipients are sterile and all culture processes aseptic.

EXAMPLE 1

Stock Culture: Whole broth of a pure culture of strain CLCO-002 is preserved frozen in 20% glycerol.

Inoculum: A frozen culture or a well grown slant culture (5% vol.) is used to seed 100 ml of seed medium described previously contained in a 250 cc shake flask. The flask is incubated during 48 hr. 500 ml of the same medium in 2 L Erlenmeyer flask are seeded with 10% of the first stage inoculum. The flask is incubated during 48 h.

Fermentation: With 2.5 L of second stage inoculum seed 50 L of production medium already described in a 75 L fermentation tank. The fermentation is carried out during 96 hours with 400 rpm agitation and airflow of 0.5 V/V.M.

Monitor secondary metabolite production by assay of whole broth against A-549 or by HPLC.

Isolation: 10 L of whole harvested broth was filtrated to separate the biomass and other solids. The mycelial cake was extracted twice with a mixture solvent (2.4 l) of $CHCl_3$: CH_3OH:H_2O (2:1:1), and the activity was concentrated in the lower layer. The organic solvent was concentrated and evaporated to dryness *in vacuo* to yield 3.2 g of crude extract. The extract was chromatographed on silica gel "vacuum flash" column. After washing with a mixture of n-hexane-ethyl acetate 1:1, the column was developed with an ethyl acetate-methanol gradient. The progress of the elution was checked for cytotoxicity against A-539 cells and monitored by TLC (chloroform-methanol 9:1) and analytical reverse phase HPLC-photodiode array. Further purification of active fractions (250 mg) was achieved by column chromatography on silica gel and the activity was eluted with chloroform-methanol 92:8 and 95:5. Each of these fractions were chromatographed on a column of C18 reversed phase and eluted with methanol-water 65:35 to give 12 mg of staurosporine, 4 mg of IB-97224, and 8 mg of IB-97225.

Biological activity: The antitumor cells employed have been P-388D_1 (suspension culture of a lymphoid neoplasm from DBA/2 mouse), A-549 (monolayer culture of a human macrocytic lung carcinoma), HT-29 (monolayer culture of a human

colon carcinoma), and SK-MEL-28 (monolayer culture of a human melanoma). P-388D_1 cells were seeded into 16 mm wells at $1x10^4$ cells per well in 1 ml aliquots of MEM 5FCS containing the indicated concentration of drug. A separate set of cultures without drug was seeded as control of growth to ensure that cells remained in exponential phase of growth. All determinations were carried out duplicated. After three days of incubation at 37 °C in 10% CO_2 atmosphere with 98% humidity, the IC_{50} was calculated by comparing the growth in wells with drug with the growth in control wells without the drug. A-549, HT-29, and SK-MEL-28 cells were seeded into 16 mm wells at $2x10^4$ cells per well in 1 ml aliquots of MEM 10FCS containing the indicated concentration of drug. A separate set of cultures without drug were seeded as control of growth to ensure that cells remained in exponential phase of growth. All determinations were carried out duplicated. After three days of incubation at 37°C in 10% CO_2 atmosphere with 98% humidity, the well were stained with 0.1% Crystal Violet. The IC_{50} was calculated by comparing the growth in wells with drug with the growth in control wells without the drug.

In Table 3 are presented the activity expressed as IC_{50} (μM)

TABLE 3

Cell line	IC_{50} (μM)	
	IB-97224	**IB-97225**
P388D_1	0.04	0.02
A-549	0.002	0.002
HT-29	0.004	0.004
SK-MEL-28	0.004	0.002

Cited References

The following references have been cited herein, and they are hereby incorporated herein by reference:

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Van der Auwera P., Labbe M., Mayberry W.R., Ferguson K.P., and Lambe D.W.Jr., *J. Microbiol. Methods* 4: 265-275, 1986

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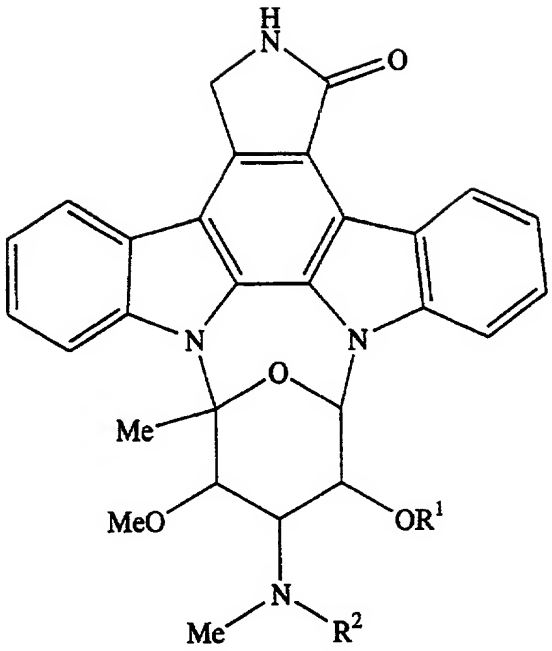
CLAIMS

1. Compounds of formula (1):



(1)

wherein:

R^1 is a hydrogen atom, an alkyl group having 1 to 6 carbon atoms or an alkoxy group having 1 to 6 carbon atoms; and

R^2 is a hydrogen atom, an alkyl group having 1 to 6 carbon atoms or an alkoxy group having 1 to 6 carbon atoms;

and pharmaceutically acceptable salts thereof.

2. A compound according to claim 1, wherein R^1 is a hydrogen atom or an alkyl group having 1 to 4 carbon atoms.

3. A compound according to claim 2, wherein R^1 is a hydrogen atom, a methyl group, or an ethyl group.

4. A compound according to claim 3, wherein R^1 is a hydrogen atom.

5. A compound according to claim 1, wherein R^2 is a hydrogen atom or an alkyl group having 1 to 4 carbon atoms.

6. A compound according to claim 5, wherein R^2 is a hydrogen atom, a methyl group, or an ethyl group.

7. A compound according to claim 6, wherein R^2 is a hydrogen atom or a methyl group.

8. A compound according to claim 1, wherein:
R^1 is a hydrogen atom or an alkyl group having 1 to 4 carbon atoms, and
R^2 is a hydrogen atom or an alkyl group having 1 to 4 carbon atoms.

9. A compound according to claim 8, wherein:
R^1 is a hydrogen atom, a methyl group, or an ethyl group; and
R^2 is a hydrogen atom, a methyl group, or an ethyl group.

10. A compound according to claim 1, wherein R^1 is a hydrogen atom and R^2 is a methyl group.

11. A compound according to claim 1, wherein R^1 and R^2 are both hydrogen atoms.

12. A process for the production of a compound of formula (1), as defined in any one of claims 1 to 11, or a pharmaceutically acceptable salt thereof, comprising cultivating a strain of a microorganism capable of producing a compound of formula (1), recovering the compound of formula (1) from the cultured broth, and, optionally, salifying the recovered compound.

13. A process according to claim 12, wherein the microorganism is an actinomycete strain.

14. A process according to claim 13, wherein the microorganism is the actinomycete strain CLCO-002 (CECT-3347)

15. A pharmaceutical composition containing as an active ingredient a compound of formula (1) as defined in any one of claims 1 to 11, or a pharmaceutically acceptable salt thereof, in conjunction with a pharmaceutically acceptable carrier or diluent.

16. A compound of formula (1) as defined in any one of claims 1 to 11 or a pharmaceutically acceptable salt thereof for use as a medicament.

17. The use of a compound of formula (1) as defined in any one of claims 1 to 11 or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the treatment or prophylaxis of malignant tumours in a mammal.

18. A method for the treatment or prophylaxis of malignant tumours in a mammal, comprising administering to a mammal in need of such treatment an effective amount of a compound of formula (1) as defined in any one of claims 1 to 11 or a pharmaceutically acceptable salt thereof.

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,

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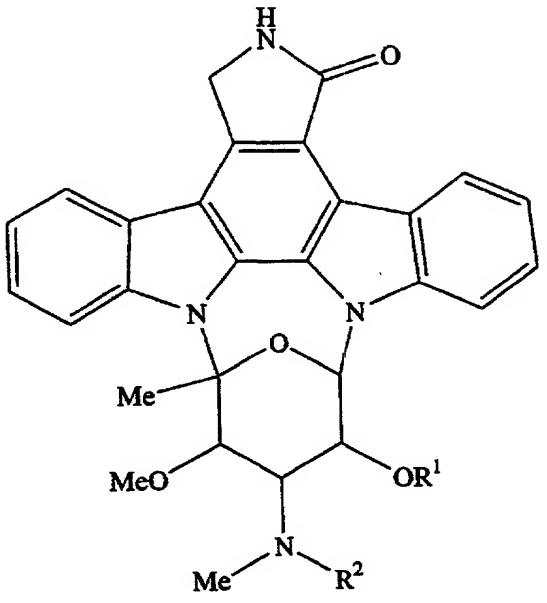
(54) Title: NEW INDOLOCARBAZOLE ALKALOIDS FROM A MARINE ACTINOMYCETE



(1)

(57) Abstract: The invention provides compounds of formula (1) wherein R^1 is a hydrogen atom, an alkyl group having 1 to 6 carbon atoms or an alkoxy group having 1 to 6 carbon atoms; and R^2 is a hydrogen atom, an alkyl group having 1 to 6 carbon atoms or an alkoxy group having 1 to 6 carbon atoms; and pharmaceutically acceptable salts thereof. The invention also relates to a process for obtaining the compounds, compositions containing them and their therapeutic use. The compounds display excellent activity against mammalian cancer cell lines.

Document #: 776496

Attorneys' Docket No. _____

USA

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: New Indolocarbazole Alkaloids From A Marine Actinomycete

which is described and claimed in:

[ ] the attached specification

[ ] the specification in application Serial No. _____ filed _____

[x] PCT International Application No. PCT/GB00/02473 filed 28-Jun-2000

(if applicable) and amended on _____ under Article 19 PCT

and on _____ under Article 34 PCT

I hereby state that I have reviewed and understand the contents of the above-identified application specification, including the claims, as amended by any amendment specifically referred to herein.

I acknowledge the duty to disclose all information known to me that is material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119 of any foreign applications(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Number	Country	Date Filed	Priority Claimed
9915069.0	United Kingdom	28-Jun-1999	[x]yes [ ]no

I hereby claim the benefit under Title 35, United States Code §1.20, of the United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information that is material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56, and which became available to me between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint George Gottlieb (Reg.No.22,035); Michael I. Rackman (Reg.No.20,639); James Reisman (Reg.No. 22,007); Barry A. Cooper (Reg.No.25,204); David S. Kashman (Reg. No. 28,725); Allen I. Rubenstein (Reg.No. 27,673); Jeffrey M. Kaden (Reg.No. 31,268); Amy B. Goldsmith (Reg.No. 33,700); Norbert P. Holler (Reg.No.17,816); Tiberiu Weisz (Reg. No. 29,876); Maria A Savio (Reg No. 31,565) & Raymond B Churchill, Jr. (Reg No. 44,617) jointly, and each of them severally, my attorneys and attorney, with full power of substitution, delegation and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent and to transact all business in the Patent and Trademark Office connected therewith. Please direct all correspondence and telephone calls to:

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Signature of fifth inventor:	Fernando	Date: 13 March, 2002